Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for the Phenotypic Examination of Pseudorabies Virus for Thymidine Kinase Activity by a Plaque Selection Method

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1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) is an *in vitro* assay method which uses a selective media in a cell culture system to detect the presence or absence of extraneous thymidine kinase (TK)-positive pseudorabies virus (PRV) in a thymidine kinase-negative (TK-), modified-live PRV vaccine.

1.2 Keywords

Pseudorabies virus, PRV, thymidine kinase, TK, plaque, *in vitro*

2. Materials

2.1 Equipment/instrumentation

2.1.1 Water bath[1]

2.1.2 Incubator,[2] 36° ± 2°C, high humidity, 5 ± 1% CO_2, meeting the requirements of the current version of GDOCSOP004

2.1.3 Microscope,[3] inverted light

2.1.4 Microscope,[4] ultraviolet (UV) light

2.1.5 Pipettor[5]

2.1.6 Freezer,[6] ultra-low

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[1] Cat. No. 15-461-10, Fisher Scientific Co., 319 West Ontario, Chicago, IL 60610 or equivalent

[2] Model 3326, Forma Scientific, Inc., P.O. Box 649, Marietta, OH 45750-0649 or equivalent

[3] Model CK, Olympus America, Inc., 2 Corporate Center Dr., Melville, NY 11747-3157 or equivalent

[4] Model BH2, Olympus America, Inc. or equivalent

[5] Cat. No. P-200, Rainin Instrument Co., P.O. Box 4026, Mack Rd., Woburn, MA 01801-4628 or equivalent

[6] Cat. No. ULT1875-5-A, Revco Scientific, 275 Aiken Rd., Asheville, NC 28804 or equivalent

2.2 Reagents/supplies

2.2.1 Connective tissue, mouse, TK mutant [L-M(TK-)] cells[7]

2.2.2 Madin-Darby bovine kidney (MDBK) cells[8]

2.2.3 PRV Reference, Shope strain[9]

2.2.4 Minimum essential medium (MEM)

2.2.4.1 9.61 g MEM with Earle's salts without bicarbonate[10]

2.2.4.2 2.2 g sodium bicarbonate ($NaHCO_3$)[11]

2.2.4.3 Dissolve with 900 ml deionized water (DW).

2.2.4.4 Add 5.0 g lactalbumin hydrolysate or edamine[12] to 10 ml DW. Heat to 60° ± 2°C until dissolved. Add to **Section 2.2.4.3** with constant mixing.

2.2.4.5 Q.S. to 1000 ml with DW; adjust pH to 6.8-6.9 with 1N hydrochloric acid (HCl).[13]

2.2.4.6 Sterilize through a 0.22-µm filter.[14]

2.2.4.7 Aseptically add:

1. 100 units/ml penicillin[15]

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[7] ATCC CCL-1.3, American Type Culture Collection (ATCC), 12301 Parklawn Dr., Rockville, MD 20852-1776

[8] ATCC CCL-22, ATCC

[9] Reference quantities available upon request from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010 or equivalent

[10] Cat. No. 410-1500EF, Life Technologies, Inc., 8400 Helgerman Ct., Gaithersburg, MD 20884 or equivalent

[11] Cat. No. S-5761, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent

[12] Edamine, Cat. No. 59102, Sheffield Products, P.O. Box 630, Norwick, NY 13815 or equivalent

[13] Cat. No. 9535-01, J.T. Baker, Inc., 222 Red School Ln., Phillipsburg, NJ 08865 or equivalent

[14] Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent

[15] Cat. No. 0049-0530-28, Schering Laboratories, 2000-T Galloping Hill Rd., Kenilworth, NJ 07033 or equivalent

2. 50 µg/ml gentamicin sulfate[16]

3. 100 µg/ml streptomycin[17]

2.2.4.8 Store at 4° ± 2°C.

2.2.5 Growth Medium

2.2.5.1 900 ml of MEM

2.2.5.2 Aseptically add:

1. 100 ml gamma-irradiated fetal bovine serum (FBS)

2. 10 ml L-glutamine[18]

2.2.5.3 Store at 4° ± 2°C.

2.2.6 Hypoxanthine, aminopterin, thymine (HAT) Medium

2.2.6.1 200 ml Growth Medium

2.2.6.2 2 ml HAT media supplement (50X)[19]

2.2.7 0.01 M Phosphate buffered saline (PBS)

2.2.7.1 1.19 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4)[20]

2.2.7.2 0.22 g sodium phosphate, monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$)[21]

2.2.7.3 8.5 g sodium chloride (NaCl)[22]

2.2.7.4 Q.S. to 1000 ml with DW.

2.2.7.5 Adjust pH to 7.2-7.6 with 0.1 N sodium hydroxide (NaOH)[23] or 1 N HCl.

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[16]Cat. No. 0061-0464-04, Schering Laboratories or equivalent
[17]Cat. No. S-9137, Sigma Chemical Co. or equivalent
[18]L-glutamine-200 mM (100X), liquid, Cat. No. 320-503PE, Life Technologies, Inc. or equivalent
[19]Cat. No. H 0262, Sigma Chemical Co. or equivalent
[20]Cat. No. S 0876, Sigma Chemical Co. or equivalent
[21]Cat. No. S 9638, Sigma Chemical Co. or equivalent
[22]Cat. No. S 9625, Sigma Chemical Co. or equivalent
[23]Cat. No. 925-30, Sigma Chemical Co. or equivalent

2.2.7.6 Sterilize by autoclaving at 15 psi, 121° ± 2°C for 35 ± 5 min.

2.2.7.7 Store at 4° ± 2°C.

2.2.8 80% Acetone

2.2.8.1 80 ml acetone[24]

2.2.8.2 20 ml DW

2.2.8.3 Store at room temperature (RT) (23° ± 2°C).

2.2.9 Tissue culture flask,[25] 25 cm^2

2.2.10 Pipette, 10 ml[26]

2.2.11 Syringe, 10 ml[27] and needle, 20 ga x 1½ in[28]

2.2.12 Swine anti-PRV fluorescein isothiocyanate-labeled conjugate[29] (PRV Conjugate)

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have experience in aseptic techniques and cell culture growth and maintenance.

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[24]Cat. No. A 6015, Sigma Chemical Co. or equivalent
[25]Falcon® 3024, Becton Dickinson Labware, 1 Becton Dr., Franklin Lakes, NJ 07417 or equivalent
[26]Falcon® 7530, Becton Dickinson Labware or equivalent
[27]Luer-Lok®, Cat. No. 309604, Becton Dickinson Labware or equivalent
[28]Cat. No. 250107, Becton Dickinson Labware or equivalent
[29]Available upon request from the CVB-L or equivalent

3.2 Preparation of equipment/instrumentation

On the day of testing, set a water bath at 36° ± 2°C.

3.3 Preparation of reagents/control procedures

3.3.1 A day prior to test initiation and a day prior to each of the 3 additional L-M(TK-) passages, seed 25-cm^2 flasks with L-M(TK-) cells, in Growth Medium, at a cell count that will produce a monolayer after 24 ± 6 hr of incubation. Cells used for seeding should be from monolayers that had been previously passaged every 3 to 4 days. Three L-M(TK-) control flasks and 1 L-M(TK-) flask are required for each Test Serial. Incubate at 36° ± 2°C in a CO_2 incubator.

3.3.2 A day before the last passage, seed 25-cm^2 flasks with MDBK cells, in Growth Medium, at a cell count that will produce a monolayer after 24 ± 6 hr of incubation. Three MDBK control flasks and 1 MDBK flask are required for each Test Serial. Incubate at 36° ± 2°C in a CO_2 incubator.

3.3.3 PRV Positive Control. On the day of test initiation, rapidly thaw a vial of PRV Reference in a 36° ± 2°C water bath. Dilute the PRV Reference in MEM to contain 10^4 50% tissue culture infective dose ($TCID_{50}$)/100 µl.

3.3.4 Working PRV Conjugate. On the day of the fluorescent antibody (FA) Confirmatory Test, dilute the PRV Conjugate in PBS according to the CVB-L supplied Reference and Reagent Sheet or per the manufacturer’s recommendation.

3.4 Preparation of the Test Serial

On the day of test initiation, using a 10-ml syringe and needle, rehydrate a vial of the Test Serial with the supplied diluent. Incubate for 15 ± 5 min at RT.

4. Performance of the assay

4.1 First passage on L-M(TK-) cells

4.1.1 On the day of test initiation, decant Growth Media from all the L-M(TK-) flasks **except 1**, which remains unopened as a cell control. Label the unopened flask “Cell Control.”

4.1.2 The initial test of a Test Serial will be with a single vial (a single sample from 1 vial). Inoculate 1.0 ml of the Test Serial into an L-M(TK-) flask labeled with the Test Serial identification.

4.1.3 1.0 ml of PRV Positive Control is inoculated into an L-M(TK-) flask labeled “PRV Positive Control.”

4.1.4 1.0 ml of HAT Medium is inoculated into an L-M(TK-) flask labeled “Medium Control.”

4.1.5 Allow inoculums to absorb by incubating flasks at 36° ± 2°C for 60 ± 10 min in a CO_2 incubator.

4.1.6 After incubation, add 9.0 ml of HAT Medium to all flasks except the Cell Control (remains unopened). Incubate all flasks at 36° ± 2°C for 96 ± 24 hr in a CO_2 incubator.

4.1.7 During incubation, periodically observe all flasks for bacterial or fungal contamination. It is not necessary for microscopic observation of the L-M(TK-) cells. However, on examination, the L-M(TK-) cells will be rounded due to the HAT Medium. The Cell Control flask should remain normal.

4.1.8 After incubation, all flasks are frozen at -70° ± 5°C for at least 2 hr. Flasks may be held at -70° ± 5°C until the next passage. The Cell Control flask is discarded, as a new Cell Control flask will be used with each passage.

4.1.9 Thaw all the remaining flasks, with frequent shaking, at RT until completely thawed.

4.2 Repeat **Sections 4.1.1 through 4.1.9** for a total of 4 passages, inoculating each flask with 1.0 ml of the thawed cell and media suspension of the appropriate control or Test Serial from the previous passage instead of the initial inoculums.

4.3 Passage on MDBK cells

4.3.1 From the last thawed passage (4th passage) on L-M(TK-) cells, repeat **Sections 4.1.1 through 4.1.3**, except substitute MDBK cells for the L-M(TK-) cells and inoculate each flask with 1.0 ml of the 4th passage cell and media suspension of the appropriate control or Test Serial instead of the initial inoculums.

4.3.2 Allow inoculums to absorb by incubating at 36° ± 2°C for 60 ± 10 min in a CO_2 incubator.

Note: Do not use HAT Medium.

4.3.3 After incubation, add 9.0 ml of Growth Medium to all flasks except the Cell Control (remains unopened). Incubate at 36° ± 2°C for 96 ± 24 hr in a CO_2 incubator.

4.3.4 Observe all flasks daily with the inverted light microscope for typical PRV CPE. The CPE of PRV is visible as grape-like clusters of rounded cells in the cell monolayer. The PRV Positive Control flask should show typical PRV CPE after 96 ± 24 hr. Any Test Serial observed to have typical PRV CPE is considered a Suspect Test Serial.

4.4 Confirmation of PRV CPE in Suspect MDBK Test Serial flasks (FA Confirmatory Test). Conduct an FA Confirmatory Test using specific PRV conjugate on any Suspect Test Serial. The PRV Positive Control flask, the HAT Medium flask, and the Cell Control flask are similarly examined and

used as controls. **If CPE is not observed in a Test Serial, this procedure is not required.**

4.4.1 Decant Growth Medium from the MDBK flasks for the Suspect Test Serial(s), the PRV Positive Control, the HAT Medium, and the Cell Control (FA Test) flasks.

4.4.2 Rinse the FA Test flasks with PBS; decant the liquid.

4.4.3 Fill the FA Test flasks with 80% Acetone and incubate at RT for 15 ± 5 min.

4.4.4 Decant the 80% Acetone from the FA Test flasks and air dry at RT.

4.4.5 Pipette 2 ml of working PRV Conjugate into the FA Test Flasks and incubate for 45 ± 15 min at 36° ± 2°C in a CO_2 incubator.

4.4.6 Add 20 ml of PBS to the FA Test flasks and rotate back and forth to rinse the cells.

4.4.7 Repeat for a total of 5 washes.

4.4.8 Rinse the FA Test flasks in DW, decant, and allow to air dry or dry at 36° ± 2°C.

4.4.9 Examine the FA Test flasks with a UV microscope.

4.4.10 A flask is considered positive for PRV if typical, nuclear, apple-green fluorescence is observed in any cell.

4.4.11 For a valid FA test, the PRV Positive Control must show typical, apple-green fluorescent infected cells.

4.4.12 For a valid FA test, the HAT Medium flask and the Cell Control flask must not show fluorescence.

4.4.13 If either **Section 4.4.12 or Section 4.4.13** criteria are not met, the test is considered a **NO TEST** and the entire test, starting with a new vial of the Test Serial, is repeated.

5. Interpretation of the test results

5.1 For a valid test, the HAT Medium flask and MDBK Cell Control must remain free of CPE and all flasks must remain free of bacterial or fungal contamination.

5.2 The PRV Positive Control must show CPE after passage in the MDBK cells.

5.3 For a **SATISFACTORY** test, a Test Serial must not show CPE after passage in MDBK cells.

5.4 Any Test Serial exhibiting typical PRV CPE in the initial test and found positive in a valid FA Confirmatory Test is retested in duplicate using 2 new vials of the Test Serial.

5.4.1 If either of the 2 retests of the Test Serial exhibits typical PRV CPE and is found positive in a valid FA Confirmatory Test, the Test Serial is **UNSATISFACTORY**.

5.4.2 If no typical PRV CPE is observed in either of the 2 retests, the Test Serial is **SATISFACTORY**.

5.5 Any Test Serial exhibiting typical PRV CPE in the initial test and found negative in a valid FA Confirmatory Test is considered **INCONCLUSIVE** for TK- activity. Repeat **Section 4.3.1** and examine for possible viral contamination.

6. Report of test results

Record all test results on the test record.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.318, U.S. Government Printing Office, Washington, DC, 2000.

7.2 Kit, S and Qavi, H. Thymidine kinase (TK) induction after infection of TK-deficient rabbit cell mutants with bovine herpesvirus type I (BHV-1): isolation of TK- BHV-1 mutants. Virol 1983; 130(2):381-389.

8. Summary of revisions

This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. The following is a list of significant changes made from the superseded protocol:

8.1 The size of the tissue culture flask was changed to 25 cm^2 from 75 cm^2, and the media addition was adjusted accordingly.

8.2 The final passage in MDBK cells was changed to 25 cm^2 from 96-well plates.

8.3 The media formulations were updated.

8.4 The validity requirement for a predetermined titer for the PRV Positive Control was removed.